Revolutionizing Medicine: Breakthrough Cell-Based Delivery System for Targeted Therapy Unveiled.

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Table of Contents

Abstract

The global burden of cancer remains high, with tens of millions of new cases being diagnosed and millions of lives being lost to the disease each year. Chemotherapy and targeted therapies have limitations and can cause serious side effects. Nucleic acids, including DNA and RNA, offer immense potential as innovative therapeutics for the treatment of cancer and various diseases, but their delivery to the target cells of tissues is very challenging. Various carriers have been developed to enhance delivery, including lipid nanoparticles and viral vectors, but challenges such as immunogenicity and off-target accumulation remain. Cell-mediated delivery systems offer important benefits, including a longer circulation time, enhanced homing to the target site, improved biocompatibility, and the possibility of engineering the cell carriers to sense and respond to disease-associated biomarkers. This paper provides an overview of currently disclosed cell-based delivery systems and proposes improvements to address their limitations. A versatile delivery system that could revolutionize medicine by achieving targeted delivery of therapeutic nucleic acids to specific tissues while minimizing side effects is presented.

Introduction

GLOBOCAN 2020 report revealed that worldwide, approximately 19.3 million individuals were diagnosed with cancer and nearly 10.0 million lost their lives to the disease in 2020 [1]. Despite the development of numerous anticancer medications, the clinical outcome is still dire since normal cells are also killed in addition to cancer cells during treatment. Chemotherapy frequently causes systemic toxicity [2] due to its lack of selectivity, sensitivity against tumor cells, and the use of large dosages [3]. Despite being advertised as more specific toward cancer cells while sparing non-target cells, targeted therapies can still cause severe adverse-events [4–6] and have limited reach due to patient eligibility [7] and the gradual onset of resistance [8]. Therefore, to improve overall survival and patients' quality of life, developing novel therapeutic modalities for the treatment of cancer is crucial.

Nucleic acids such as DNA and RNA constitute a promising class of innovative therapeutics that address the limitations of conventional chemotherapy [9], and offer a broad range of potential applications in the treatment of viral infections [10,11], genetic diseases [12–14], autoimmune diseases [15,16], cardiovascular diseases [13,17], and cancer [12,18]. However, their successful delivery to the target is hampered by several obstacles. They can be quickly degraded by nucleases when administered directly, reducing their bio-availability in the circulation. Their accumulation inside cells is also exceedingly difficult because of their negative charge and large size, which complicate their transport across the plasma membrane.

Various carriers or vectors have been engineered to enhance nucleic acids' localization into cells tissues [13,19–22]. Despite the significant progress made in this area, many obstacles remain to be overcome [19,22,23]. For example, some carriers are immunogenic [24] or have difficulty reaching the target [25,26]; other carriers accumulate dangerously in the liver [27,28] or are rapidly cleared from the circulation [29].

Nanoparticles and monoclonal antibodies are established as highly promising carriers and targeting moiety for the delivery of therapeutics to diseased sites [19,21,30,31], but they suffer from low tumor penetrance and high off-target accumulation in various tissues when administered intravenously. A meta-analysis of 376 data sets published from 2005 to 2018 concerning nanoparticle-mediated delivery of therapeutics to solid tumors found that the mean and median delivery efficiencies were 2.23% and 0.76% of the injected dose, respectively [32]. A comparative analysis of the tissue distribution of Zirconium-89 labeled antibodies showed that 4 days post-injection, 10.3-14.2%, 4.9-7.4%, 0.9-1.8%, 1.0-1.6%, and 0.1-0.9% of the injected dose accumulate in the liver, fat tissues, spleen, kidneys, and tumors, respectively [33].

Cell-mediated delivery systems provide several benefits, such as the ability to escape the immune system, prolonged circulation times, and improved biocompatibility [34]. Furthermore, they can be engineered to sense and respond to disease-associated biomarkers [35–37], act as a platform for the production of biotherapeutics [37–39] can benefit from chemotaxis-mediated cell trafficking, which is the process of active recruitment of circulating cells to infiltrate a specific region, to enhance therapeutic efficacy and reduce side-effects by actively targeting the diseased site while minimizing payload's release in off-target regions [34,40].

This paper is aimed at presenting an overview of cell-based delivery of therapeutics as a means to address the difficult challenge of delivering therapeutics to specific sites in the body with the goal of treating diseases with significant unmet medical needs, including genetic diseases and cancer, and **to provide suggestions for improvement** after revealing the limitations of the herein-described therapeutic delivery modalities.

Calidi Biotherapeutics cell-based delivery system

Their technology is described here: https://calidibio.com/our-science
Their technology is covered by patent: https://patents.google.com/patent/US20190367880A1, and https://patents.google.com/patent/US20200140824A1/en

Oncolytic viruses are viral particles that preferentially infect and replicate inside cancer cells [41,42]. They can be administered by intravenous injection or directly injected into the tumor. However, the body's immune system considers them foreign, which leads to their inactivation and elimination [42,43]. Direct intratumoral injection delivers the oncolytic virus to the tumor site, but this method of delivery is inappropriate for difficult-to-reach tumor lesions such as those located in the central nervous system. Calidi Biotherapeutics solution to the problem consists of hiding and protecting the oncolytic viruses against the patient's immune system by loading them into stem cells. The stem cells act as a vehicle and a replication platform that deliver the oncolytic viruses to tumor sites and amplify them, respectively [44].

Replication-competent adenoviral vectors, CRAd-S-pk7, were loaded into Neural Stem Cells (NSCs) by transduction, giving NSC-CRAd-S-pk7. To limit CRAd-S-pk7 replication to cancer cells, its E1A gene is put under the control of the human surviving promoter [45]. NSC-CRAd-S-pk7s were injected 2 to 3 mm away from the tumor sites of mice bearing human glioma xenografts as well as radio-resistant glioma stem cell (GSC)-enriched xenografts. The cells were able to effectively migrate toward tumor lesions, deliver their oncolytic virus payloads to targeted glioma cells, and prolong the median survival of the treated mice [45].

NSC-CRAd-S-pk7 was evaluated in a phase 1 clinical trial to examine its safety and activity in patients with newly diagnosed high-grade gliomas [46]. The cells were injected into the walls of the resection cavity after the neurosurgical removal of the tumor. No formal dose-limiting toxicity was reached, adverse events were manageable, and treatment with NSC-CRAd-S-pk7 was concluded to be safe.

Cytonus Cargocyte™ platform

Their technology is described here: https://cytonus.com/our-science
This invention is covered by patent: https://patents.google.com/patent/WO2021158991A1/en

Mesenchymal stromal cells (MSCs) hold great promise as delivery systems because of their ability to actively home to tumor lesions [47,48]. To increase their safety profile, the nucleus can be removed to prevent their uncontrolled proliferation, avoid lung trapping [49–51], and prevent their differentiation into cancer-promoting stromal cells [52]. The enucleated cells can remain alive for days and temporarily retain their capacity to migrate toward specific regions in response to gradients of chemoattractants, and to support the translation of messenger RNAs (mRNAs) to synthesize any protein of interest [53].

Wigler and Weinstein [54] developed a rapid and effective method of obtaining enucleated cells. The cells are ultracentrifuged in a Ficoll gradient containing Cytochalasin B, a metabolite isolated from the mold *Helminthosporium dematioideum* that was found to induce spontaneous nuclear extrusion in cultured mouse fibroblast cells [55].

Before enucleation, the expression of certain surface proteins, such as endothelial adhesion molecule ligands and chemokine receptors, can be increased by transducing the MSCs with lentiviral vectors encoding the corresponding genes [53]. Chemokine receptors confer the delivery system with the capacity to migrate toward a specific site or tissue by following chemokine gradients. To reach specific tissues, the delivery system must exit the blood vessel by interacting with endothelial adhesion molecules expressed on the surfaces of endothelial cells.

Synthetic mRNAs can be loaded into the enucleated cells by lipofection [53]. The mRNA could encode cytoplasmic or secreted therapeutic proteins such as immunomodulatory cytokines (IL-12, IL-10), immune checkpoint inhibitors, gene-editing technology (CRISPR-Cas9), or prodrug-activating enzymes. More complex payloads, including drug-loaded nanoparticles and oncolytic viruses, could also be transported by cargocytes.

Cargocytes are delivery systems with a defined lifespan of around 72 hours that can migrate toward target tissues by following a gradient of chemokines, and transiently express and secrete an mRNA-encoded therapeutic payload at the diseased site [53]. Multiple timely intravenous injections will be required for a sustained therapeutic effects.

SEAKER platform

This invention is covered by patent: https://patents.google.com/patent/WO2019006464A1/en
A simpler description of the system is available here: https://doi.org/10.1038/s41589-021-00933-0

The apparition of resistance is an inherent problem with Chimeric Antigen Receptor (CAR) cell therapy. CAR T-cells only kill cells that bear a specific target antigen on their surface. Cancer cells that harbor an altered version of the target antigen will escape the onslaught. SEAKER is an acronym for Synthetic Enzyme-Armed KillER cells, a group of engineered CAR T-cells that constitutively express and secrete prodrug-activating enzymes in the circulation and at tumor sites [56].

Primary human T-cells or Jurkat T-cells are transduced with lentiviral vectors that encode *Pseudomonas sp.* carboxypeptidase G2 (CPG2) or *Enterobacter cloacae* β-lactamase (β-Lac) genes and a CAR cassette, separated by a P2A self-cleaving peptide [56]. Upon stimulation by antigen-presenting target cells, the CAR T-cells undergo clonal expansion, leading to a significant increase in CPG2 or β-Lac production and secretion [56]. β-Lac is an enzyme that converts the prodrug Ceph-AMS into the highly cytotoxic drug 5´-O-Sulfamoyladenosine (AMS), and CPG2 is an enzyme that converts the prodrug AMS-Glu into AMS [56].

Intra-peritoneal or intravenous injection of SEAKER cells results in their localization and the production of enzymes at the engrafted tumor sites. The intravenous injection of prodrug, days after cell administration, showed a significant increase in survival compared to mice treated with SEAKER cells alone and untreated control mice [56].

Regulated Viral Vector Delivery System (RVVDS)

This invention is covered by patent: https://patents.google.com/patent/US20220136004A1/en

Gene-editing systems such as CRISPR-Cas must access the target cell's genome to perform their function. Viral and non-viral delivery systems are being deployed to localize them inside the cells' cytoplasm or nucleus, but they face immune resistance and lack specificity when applied in vivo. As a result, the success rate of gene editing is low, and the treatment toxicity is unacceptably high.

To address the aforementioned problems, engineered cells that can home, produce, and release viral vectors in specific tissues of an animal were developed. In certain aspects of their invention, the engineered cells comprise a vector encoding the E1A gene of Adenovirus, an adenoviral amplicon genome encoding a Genome Modification System (GMS), and a helper adenoviral genome encoding genes required for the packaging of the amplicon inside of an adenoviral vector. The E1A gene can be under the control of a tetracycline- or doxycycline-inducible promoter, or under the transcriptional activation of the Intra-Cellular Domain (ICD) of a synthetic notch (SynNotch) receptor.

The engineered cells are injected intravenously and migrate to a location containing the target cells. In the presence of inducing signals (a membrane-bound antigen engaged by the ligand-binding domain of the SynNotch or the small molecule doxycycline), E1A proteins are produced, and viral vector production is initiated. The engineered cells eventually die and release the viral vectors in the milieu, which will transduce the surrounding target cells with the Genome Modification System.

NFAT-Cre-CAR-T cell containing HSV-1 type oncolytic virus

This invention is covered by patent: https://patents.google.com/patent/CN111676245B/en

Chimeric Antigen Receptor (CAR) T-cell therapies have limited activity in the treatment of solid malignancies [57], and oncolytic viruses can be neutralized by the immune system when administered systemically. To combine the advantages of both treatments, an engineered cell and a recombinant oncolytic virus, NFAT-Cre-CAR-T cell and Switch-oHSV-1-X, respectively, were invented. This system is similar in principle to a previously described replication-defective oncolytic adenoviral vector that is rendered replication-competent by the expression of a Flp recombinase, which switches-on EA1 gene transcription by excising a transcriptional STOP cassette flanked by two FRT sites, and inserted between a strong CMV promoter and the E1A coding sequence [58].

The NFAT-Cre-CAR-T cell comprises a Cre recombinase gene under the control of a NFAT, NF-κB and/or AP-1 promoter, and a Chimeric Antigen Receptor-encoding gene whose expression allows the cell to recognize and kill target cells bearing a specific antigen on their surface. Switch-oHSV-1-X is a recombinant Herpes Simplex Virus type 1 (HSV-1) that comprises an ICP4 gene under the control of a constitutive CMV promoter and whose transcription is prevented by a STOP cassette (Loxp-mCherry-polyA-Loxp) inserted between the promoter and the coding sequence. The structure of the ICP4 gene is thus CMV-Loxp-mCherry-polyA-Loxp-**ICP4**-polyA.

Switch-oHSV-1-X viral particles are produced by transfecting a linearized oHSV-1-X containing a native ICP4 gene and a linearized recombination plasmid containing mCherry-polyA-Loxp-**ICP4**-polyA, into Vero-ICP4 cells. The native ICP4 gene is exchanged with the mCherry-polyA-Loxp-**ICP4**-polyA cassette by homologous recombination, which is revealed by the formation of red lytic plaques. Purified and concentrated Switch-oHSV-1-X viral particles are loaded into NFAT-Cre-CAR-T cells by co-incubation. Positively transduced cells are further isolated by flow cytometry.

When oncolytic virus-loaded NFAT-Cre-CAR-T cells home to tumor sites and engage their targets through their CAR receptors, NFAT is up-regulated and induces the expression of Cre recombinase proteins, which in turn bind to their LoxP attachment sites to mediate the excision of the Loxp-mCherry-polyA-Loxp cassette, leading to the constitutive expression of ICP4, a protein that is indispensable for the replication of the oncolytic HSV-1. The localized production and release of oncolytic HSV-1 at the tumor sites combined with the cancer cell-killing activities of the NFAT-Cre-CAR-T cells should enhance the therapeutic efficacy of the system and improve safety.

scFv-synNotch-Cre-CAR-T cell containing HSV-1 type oncolytic virus

This invention is covered by patent: https://patents.google.com/patent/CN111676199B/en

This invention is similar to "Regulated Viral Vector Delivery System" but utilizes a synthetic notch receptor that includes a Cre recombinase as the intracellular domain (ICD) and Switch-oHSV-1-X as the payload. The viral protein ICP4 is prevented from being transcribed by a STOP cassette flanked by loxP sites. When the synthetic notch receptor's scFv domain binds its target antigen, the Cre recombinase is excised, translocates into the nucleus, and excises the STOP cassette, activating the expression of ICP4, which triggers Switch-oHSV-1-X replication.

A cassette that comprises a CAR gene, a P2A sequence, and a scFv-synNotch-Cre is cloned into a transposon plasmid. scFv-synNotch-Cre-CAR-T cells are generated by transfecting T cells with a mixture of a scFv-synNotch-Cre transposon and a transposase expression vector. Switch-oHSV-1-X is loaded by incubating the scFv-synNotch-Cre-CAR-T cells with the viruses. Oncolytic virus-loaded cells are further isolated by flow cytometry.

Chemotherapeutic-loaded cells

Chemotherapeutic drugs can be delivered by taking advantage of the homing capacity of immune cells to sites such as inflamed tissues or tumor lesions. To minimize drug release, increase drug loading, and enhance carrier cell survival, doxorubicin was condensed with silica to form Dox-silica nanocomplexes, and then incubated with RAW264.7 cells (a murine macrophage cell line) for two hours. After intravenous injection, the macrophages accumulate in the lung, then gradually migrate and release doxorubicin into tumors, leading to a significant increase in the survival of the treated tumor-bearing mice [59]. In a similar approach, the cytotoxic drug Paclitaxel (PTX) was formulated as PTX loaded nanoparticles and incorporated into mesenchymal stem cells (MSCs) by endocytic uptake [60]. The loading capacity is approximately 4 pg of paclitaxel per cell. The PTX-containing MSCs demonstrated enhanced tumor accumulation and retention capabilities due to increased ROS production and the upregulation of the chemokine receptor CXCR4 [60]. Intravenously injected PTX-loaded MSCs accumulate in lung tumors and are able to deliver more than 1000ng of PTX per gram of tumor, resulting in improved anti-cancer efficacy, a significant reduction in tumor burden, and increased survival [61].

Cytotoxic chemotherapeutic payloads can be attached to the surface of tumor-homing cells for successful delivery into difficult-to-access sites. This approach was investigated for the delivery of SN-38 to tumor-bearing lymphoid organs [62]. The delivery system is T cells that were activated and allowed to proliferate in the presence of IL-2 and the mTOR inhibitor rapamycin to preserve CD62L and CCR7 expression, both of which are required for efficient lymph node homing. SN-38 is a very potent, highly hydrophobic topoisomerase I inhibitor with a very poor pharmacokinetic profile [63]. The drug is retained in multilamellar lipid nanocapsules and stably conjugated to T cell surface proteins, giving SN-38 NC-T cells and providing a loading capacity of approximately 0.4 pg of SN-38 per cell [62]. Eμ-myc tumor-bearing mice treated with the cells had a significant reduction in tumor burden and showed significant increases in survival [62].

Recombinant proteins-producing cells

Chemotherapy drugs are highly cytotoxic compounds that can cause serious tissue and organ damage when administered systemically. Prodrugs are somewhat inert compounds that are converted into cytotoxic products by proteins called prodrug-activating enzymes, which are delivered at tumor sites by tumor-tropic stem cells or immune cells.

HB1.F3.C1 cells were transduced with replication-deficient adenovirus, so they constitutively express and secrete rabbit carboxylesterase (rCE), an enzyme that converts irinotecan to its active cytotoxic metabolite SN-38 [47]. The rCE-producing HB1.F3.C1 cells were intravenously injected into a mouse model of metastatic neuroblastoma. The injected cells were able to localize to tumor sites regardless of their location, and the systemic administration of a prodrug, CPT-11, allowed the survival of all treated mice for more than 6 months [47]. A similar approach using the enzymes cytosine deaminase and thymidine kinase delivered by human stem cells to metastatic lung cancers for the local activation of the systemically administered prodrugs 5-fluorocytosine and ganciclovir, was shown to significantly improve mice's survival over controls and decrease tumor burden [48].

Another approach to localized drug delivery is the direct transplantation of therapeutic cells into the diseased site. Human Embryonic Kidney cells (HEK-293T) were engineered with a synthetic gene circuit to sense and respond to both the levels of disease biomarkers Interleukin 22 (IL-22) and Tumor Necrosis Factor (TNF) by triggering the production of anti-inflammatory therapeutics IL-4 and IL-10 [37]. The designer cells were formulated with alginate-(poly-L-lysine)-alginate capsules and intraperitoneally implanted in mouse models of imiquimod-induced psoriasis-like lesions. The implants attenuated the burden of psoriasis in mice and prevented psoriasis-like plaque formation and the onset of psoriatic flares [37].

SynNotch is a synthetic receptor comprising a modular extracellular domain that binds a target ligand and an intracellular transcriptional domain that enables customized user-defined input and output functions when expressed by mammalian cells [35]. This system was exploited to develop a cell-based sensor (α-HBs SNR Cell) that produces antiviral therapeutics upon detecting the presence of hepatitis B virus (HBV) or membrane-bound HBV antigens [64]. An intracellular Gal4-VP64 transcriptional activator domain is excised from the SynNotch upon ligand engagement and drives the expression of antiviral interferon-beta (IFNβ) or anti-HBV antibodies. When co-cultured with target antigen-bearing cells or HBV virion-producing cells, the α-HBs SNR cells secreted a significant amount of IFNβ or anti-HBV neutralizing antibodies [64].

Bioengineered immune cells

Chimeric Antigen Receptors (CAR) are synthetic surface proteins composed of an extracellular antigen recognition domain, a transmembrane domain, and an intracellular domain that activate downstream signaling leading to cell proliferation, survival, and differentiation upon induction. When expressed by cytotoxic immune cells such as T lymphocytes or Natural Killer cells, they allow the specific recognition of antigen-bearing target cells, which are engaged and killed by immunological synapse-delivered cytotoxic proteins (granzymes and perforins).

Although Chimeric Antigen Receptors (CAR) T-cells have revolutionized the treatment of a subset of hematological malignancies [65], their adoption in the treatment of patients with solid tumors is still hampered by challenging obstacles [57,66]. Numerous approaches are currently being proposed and explored to overcome the identified problems and to improve the antitumor activity and safety profile of CAR-engineered immune cells [57,67], including their combination with oncolytic virus therapy [68]; the use of logic gating to increase their safety (ON switched CARs), cancer-cells discrimination capability (AND-gating), or improve eradication for heterogeneous tumors (OR-gating) [69]; their engineering to express prodrug-activating enzymes that activate cytotoxic drugs at the tumor sites, enhancing anticancer activity [56]; their engineering to produce allogeneic, hypoimmunogenic CAR T cells, which are administrable to multiple patients and less likely to trigger Graft versus Host Disease (GVHD) [70]; and their engineering to attenuate exhaustion or senescence to improve their proliferative capacity and effector function [71].

SiVEC Biotechnologies BactPac™

Their technology is described here: https://www.sivecbiotechnologies.com/technology, https://www.sivecbiotechnologies.com/applications
Their inventions are covered by patents: https://patents.google.com/patent/WO2018187381A3/en, https://patents.google.com/patent/US20220389462A1/en

Their invention concerns the use of engineered bacterial cells to produce and deliver gene-encoded therapeutics to targeted epithelial cells in tissues. *E. coli* strain SVC1 was engineered to express short-hairpin RNA (shRNA) payloads and *Yersinia pseudotuberculosis* invasin (inv), a protein that interacts with b1 integrin on the surface of target epithelial cells and leads to the bacteria internalization by receptor-mediated phagocytosis [72]. The bacteria are auxotrophic for diaminopimelic acid (DAP), which is indispensable for bacterial cell-wall integrity [73] and absent in eukaryotic cells, causing them to become instable, rapidly lyse, and release their content inside the phagosome. The bacteria express hemolysin HlyA, a protein that prevents phagosome maturation [74] and causes its lysis [75], resulting in the shRNA payloads localization in the host's cytoplasm [76]. Beyond shRNA, the engineered SVC1 bacteria can produce and deliver circular and linear messenger RNAs (mRNAs), gene-editing ribonucleoprotein complexes (CRISPR-Cas + guide RNA), and therapeutic proteins [76]. In vivo studies on mice showed that the delivery system was well tolerated, minimally immunogenic, and able to localize to a broad range of epithelial tissue types [76].

Suggestions for improvement

The technical problem that the afore-mentioned technologies are trying to address concerns the effective delivery of therapeutics to specific sites of the body to elicit localized therapeutic effects while minimizing side effects. After outlining the limitations of the described drug delivery modalities, I will propose some suggestions for improvement and introduce a cutting-edge delivery system that I am currently developing.

After intravenous injection, mesenchymal stem/stromal cells (MSCs) are trapped in the lung because of their large size [50,51] and the expression of integrins [77]. MSCs homing to tumor lesions is a very inefficient process [78]. Engineered MSCs that express CXCR4 can home to sites of injury or inflammation instead of tumors [79]. After reaching tumor sites, MSCs can support their progression by promoting angiogenesis [80] and differentiating into tumor-associated stromal cells [52]. When used as delivery systems for oncolytic viruses, insufficient tumor homing after intravenous injection [78,81,82] and the long delay between manufacture and administration into patients can become unacceptable [43], although this could be solved with the use of allogeneic MSCs. How can we improve the safety features of MSCs while keeping their advantages as a delivery system? Oncolytic virus-loaded MSCs can be administered around a resected tumor lesion to limit their systemic spread and improve tumor homing [46,81] or engineered to express an inducible safety system that causes cell death when treated with inducing small-molecule ligands [83] or a regulatory-approved monoclonal antibody [84].

A clever solution to prevent MSCs proliferation, entrapment in the lung, and unsolicited differentiation into tumor-associated stromal cells consists of removing their nucleus. The resulting enucleated cells can translate messenger RNAs (mRNAs), migrate toward a target site in response to a chemokine gradient, and exit the blood vessel to infiltrate tissue [53], but they will not be able to transcribe genes. As a consequence, they cannot respond to disease-associated biomarkers, even if they are able to sense them. Another limitation concerns the constitutive expression of the transfected synthetic RNAs that causes the enucleated cells to produce and accessoryly secrete the payloads when they are still in the circulation. In addition, payloads such as shRNAs or gene-editing systems must access the target cells' cytoplasm or nucleus to elicit therapeutic effects. The enucleated cells can express fusogens at their surfaces, but this can lead to unwanted cell-cell fusion and death during manufacture [85], and depending on the fusogen used, the enucleated cells could be neutralized by the human serum complement [86].

Solid cancers are surrounded by a hostile tumor microenvironment (TME), and are composed of a heterogeneous population of difficult-to-access cancer cells, which can be challenging to eradicate with antigen-specific CAR T cells. In addition to their effector function, CAR T cells can serve as delivery systems for oncolytic viruses [68], or prodrug-activating enzymes (PAEs) [56] to enhance anticancer activity. Co-expression of PAEs along with a chimeric antigen receptor will lead to the continuous secretion of the enzyme when the CAR T cells are still circulating in the bloodstream. Injected prodrugs will be converted into their active forms in tissues where PAE accumulates, resulting in off-target effects that can lead to serious adverse events. How to avoid that? The solution is simple: PAE's expression should only be induced when the CAR T cells have infiltrated the tumor and are engaging their targets.

A T cell can be engineered to express synthetic receptors such as SynNotch to sense and respond to external antigens exposed on the surface of target cells. The T cell may further be transduced with viral packaging genes and a "transfer" or amplicon genome to allow the packaging of therapeutic payloads into viral vectors. To minimize the risk of viral vector production in the absence of stimuli, the use of enhanced synthetic notch receptors that are less prone to ligand-independent activation [87], and responsive promoters with low background activity to drive transgene expression [88] is highly recommended. During replication, the viral packaging genes can recombine with each other and give rise to replication-competent viruses [89–92]. Restricting the use of viral genes to the essentials, and separating these genes into multiple isolated constructs

could improve safety. In the case of adenoviruses, the packaging sequence of the helper genome could be excised by a site-specific recombinase to minimize the formation of helper viruses.

Nuclear Factor of Activated T cells (NFAT) is an example of a transcription factor that is upregulated and drives the expression of NFAT-responsive transgenes when T cells are antigen-stimulated [93]. Tumor-infiltrating lymphocytes engineered with NFAT-regulated IL-12 were found to constitutively express low levels of IL-12, suggesting that the NFAT promoter is leaky [94]. As a result, T cell carriers comprising viral genes (E1A for adenovirus, ICP4 for HSV-1) under the control of NFAT could promote oncolytic virus replication that leads to premature payload release.

Chemotherapeutic drugs can be actively targeted at tumor lesions by being formulated as a nanocomplex and loaded into tumor-tropic cells. When injected intravenously, cell carriers such as Mesenchymal Stromal Cells (MSCs) home inefficiently to tumor lesions [78,81,82] or can end up in off-target sites [79]. The large number of cell carriers required to achieve significant drug deposition in tumor lesions [62] combined with the regulatory burden associated with cell therapy manufacturing [95] could make this approach very expensive and challenging to deploy.

Bacteria are considered foreign by the immune system. When used as a delivery system, they can end up inside professional antigen-presenting cells such as macrophages and dendritic cells, which will present the bacteria-derived antigens to immune effectors in the draining lymph nodes, leading to the initiation of a pro-inflammatory immune reaction, the production of neutralizing antibodies, and the elimination of epithelial cells presenting the bacteria-derived antigens via the major histocompatibility complex (MHC) [96]. This could be very problematic because repeated administration will be necessary to maintain the therapeutic effect as some epithelial cells have a high turnover rate [97]. Knocking down [98] or sequestering [99] MHC could address this problem.

My delivery system to revolutionize medicine

A slide presentation that introduce my project is available here: https://doi.org/10.5281/zenodo.7985593

I have developed an improved and safer delivery system by taking into consideration the limitations of the currently available therapeutic delivery modalities. I always emphasize simplicity when designing this invention. For its construction, I also use components that were proven to work effectively in mammalian cells, as revealed by the corresponding literature reports. I initially developed this system as an effective means to deliver complex therapeutics to tumor lesions, but because of its versatility as a vehicle that delivers nucleic acids (DNAs or RNAs) to specific sites of the body, applications beyond the treatment of cancer are conceivable. Disclosed here is important information concerning my delivery system's working.

The solution to achieving a significant accumulation of therapeutics at a target site (tumor lesion, specific tissue) is active targeting. To this end, T cells are well suited as carrier for payloads delivery because: (a) they have a long history of clinical applications [100,101]; (b) a large body of literature are available and cover topics such as: ex vivo and in vivo T cells expansion [102,103], allogeneic T cells generation [70], engineering of T cells to improve their effector function [57,66,104], and the use of T cells as a therapeutic delivery system [56,62]; (c) they can freely circulate in the blood and lymphatic system [105], follow a gradient of chemo-attractants [105–108], extravasate from the circulatory system to infiltrate virtually any tissue of the body [108,109]; (d) and unlike mesenchymal stromal cells (15–30 μm), they are tinier (4–12 μm) and less likely to be entrapped in the lung [49,50,110].

What should we deliver to the target site? The payload can be a therapeutic protein [111], an enzyme [112,113], DNA, RNA [114], or a combination thereof, encoded as a transgene that will be transcribed and/or translated once it has access to the cytoplasm or the nucleus of the target cells. The gene can be under the

control of a strong and constitutive polymerase II promoter, or in the case of shRNAs, a polymerase III promoter, or if we want to limit expression to certain cells, a tissue- or cancer- specific promoter. More complex payloads, such as oncolytic viruses [115,116] are also supported.

How are the payloads loaded into their carriers (T cells)? (See Figure 1) Transfection or transduction can be used if the payloads are to be packaged into low-capacity viral vectors. Transduction can be used if the payloads are packaged into adenoviruses or HSV-1 because of their very large size (from 36 to 150 kb). Of course, for the purpose of viral vector production, the viral helper genome must be transfected or transduced along with the payloads [89–92,117].

How to prevent viral vector production after the successful insertion of the payloads and the packaging genes into the carriers? Caution must be taken to prevent payload packaging before the delivery system has reached the target site and is stimulated by antigens. Viral packaging is prevented by restricting the production of viral proteins indispensable for replication, such as Gag-Pol for retrovirus or lentivirus [90], E1A for adeno-associated virus [117] or adenovirus [58], and ICP4 for HSV-1 [118].

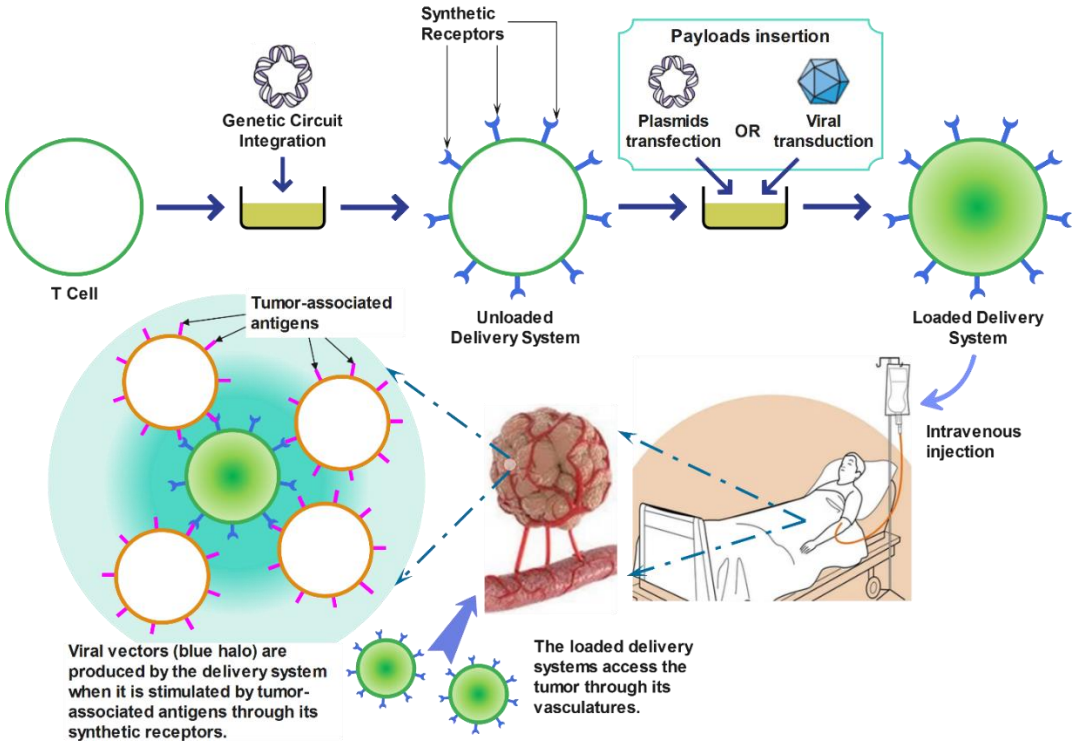

Figure 1: Generation, payloads loading, administration, and mode of action of the delivery system.

How to achieve a significant deposition of therapeutics at the target site? When the delivery system is stimulated by the right antigens, an essential viral protein is synthesized, irreversibly committing the delivery system to the production of thousands of viral vectors. The more delivery systems reach the target site, the more viral vectors will be produced. After their release, these vectors will transduce nearby cells. The transduced therapeutic transgenes can be under the control of a strong promoter that drives high levels of payload expression.

How to transfer the payloads from their carrier (the T cell) to the target? (See Figure 2) Payload transfer is accomplished by viral vectors that can localize them into the target cell's cytoplasm or nucleus. Available viral vectors are: (a) Adeno-Associated Virus (AAV) [89,117]; (b) Retrovirus or lentivirus [90]; (c) Adenovirus [91]; (d) Herpes simplex virus type 1 (HSV-1) [92].

How does the delivery system know where and when to release its payloads? (See Figure 2) Cells express on their surfaces a number of unique proteins that can serve as biomarkers. These membrane-anchored proteins can be overexpressed in certain tissues or cancers, making them ideal for identifying the target cells and thus the site where the payloads should be delivered. The delivery system senses the target cells through synthetic receptors, which, upon binding to one or more surface proteins, activate a downstream circuit that initiates payload packaging into viral vectors. In the treatment of solid cancers, the targeting of at least two surface proteins is highly advisable to limit the on-target off-tumor effect, which is the targeting of normal tissues that express the surface protein at a low level. Examples of surface proteins are: HER2 (gastric, esophageal, ovarian, endometrial, and breast cancers) [119], EGFR (gliolastoma, pancreatic, prostate, breast, colorectum, and non-small cell lung cancer) [120], ROR1 (mantle cell lymphoma, breast, ovarian, lung, and pancreatic cancer) [121,122], Claudin-6 (germ cell tumors, hepatocellular, ovarian, endometrial, and non-small cell lung cancer) [123], IL13Rα2 (pancreatic, breast, prostate, glioblastoma, and colorectal cancer) [124,125], and PSMA (prostate cancer) [126].

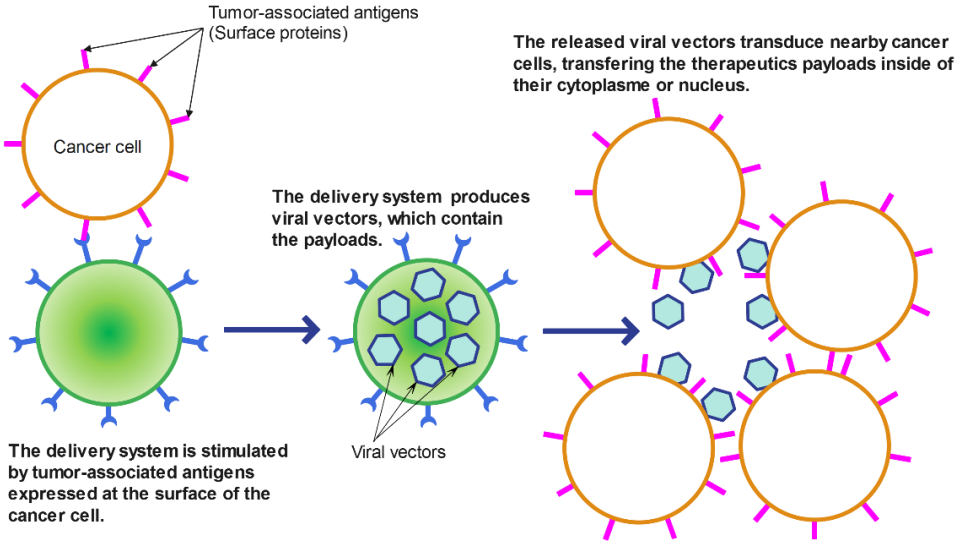

Figure 2: Mode of transfer of the therapeutic payloads from the delivery system to target cancer cells.

The particularity of my delivery system resides in an integrated genetic circuit that allows for precise target recognition, enhanced infiltration, and robust payload production upon induction. The circuit is simple and elegantly designed to minimize leakage or background transgene expression that may cause premature viral vector production. To improve safety, the risks associated with the presence of oncogenic viral genes [127], and the generation of replication-competent helper viruses [89–92] were also addressed. For the treatment of solid cancers, I have also designed a payload that can be used in combination therapy to maximize cancer cell eradication.

How to make the delivery system safer? Safety switches that cause apoptosis of the expressing cells upon the administration of an inducing small-molecule ligand can be used [83]. During apoptosis, the payloads and the DNA construct encoding the packaging genes will be degraded by endonucleases [128]. However, payloads that are already packaged into viral vectors are resistant to endonuclease degradation and can still be active. To improve specificity, two or more surface-expressed antigens can be targeted [104].

How to identify T cells that carry the payloads? The designed genetic circuit doesn't incorporate antibiotic resistance genes. Selection is achieved by Fluorescence Activated Cell Sorting (FACS).

How to deliver this therapeutic to patients? In on aspects of my invention, the loaded delivery system can be cryopreserved to facilitate its storage and shipment around the globe.

How is this delivery system going to overcome the challenges faced by cell therapy in the treatment of solid malignancies [57,66,67] **?**

- **Solid malignancies comprise a heterogeneous population of cancer cells, a dense extracellular matrix that limits immune cell infiltration, and a group of tumor-associated stromal cells that promote tumor growth.** Cancerous lesions comprise an actively proliferating rim supported by a network of blood vessels, and a necrotic core. After infiltrating the lesions and interacting with cancer cells, the delivery system begins to produce viral vectors that will transduce (infect) nearby cells. The nanometric size of the viral vectors enhances their accumulation and infiltration into the tumor lesions [129], allowing them to transduce nearby and buried cancer cells. The delivered therapeutic transgene is under the control of a tumor-specific promoter to restrict payload expression to cancer cells or a constitutive promoter to enable broad payload expression in most of the transduced cells. The payload can encode collagenase to enhance viral vector infiltration [130]. It can also encode a prodrug-activating enzyme that locally converts a prodrug into a very cytotoxic compound, enhancing its anticancer effect [47,48,56].
- **The Tumor Micro Environment (TME) is immunosuppressive**. This problem negatively impacts the effector functions of the T cells but will not impede their ability to produce viral vectors.
- **Chronic antigen stimulation and immune checkpoint inhibitors expression induce exhaustion of the T cells.** These problems only affect T cell effector functions, but won't influence viral vector production, which is robustly triggered by chronic antigen stimulation.
- **Tumor-associated stromal cells in combination with the tumor micro environment restrict cytotoxic T cells migration and infiltration.** Arming the T cells with chemokine receptors (ex: CCR4 [106], CXCR3 [107]) could address the problem by enhancing homing and infiltration into tumor lesions.
- **T cells have limited persistence in tumors.** T cells that serve as a delivery system are not required to persist in tumors. Their purpose is to produce viral vectors upon antigen stimulation. If the number of circulating delivery systems decreases, new ones can be intravenously administered.

How could this delivery system revolutionize gene therapy?

- It protects the payloads from the immune system, and will only release them once the target site is reached. That's how the notorious problems of payload clearing by the immune system and their off-target accumulation in the liver are addressed.
- It supports payloads up to 150-kb in size. Depending on the viral vectors being used, the payloads can be efficiently delivered into the cytoplasm or nucleus of the transduced cells.
- By actively homing toward the target site, it could enable effective in vivo gene corrections by delivering a sufficient amount of gene-editing systems such as CRISPR-Cas to treat most of the target cells, such as hematopoietic stem cells in the bone marrow. The payloads can be transiently expressed to limit toxicity and off-target activities.

How could this delivery system revolutionize the treatment of autoimmune diseases?

- After homing to specific sites of inflammation, the delivery system can elicit a localized therapeutic effect by secreting TGF-β [131], IL-4 [132], and/or antagonists targeting TNF-α [133,134], IL-6 [133,135], IL-7 [136], and/or IL-23 [137].
- The goal of the treatment is to avoid the risks associated with systemic immunosuppression by inducing a localized immune tolerance and attenuating the burden associated with chronic inflammation in the relevant diseased tissue.

- The delivery system can produce viral vectors that transfer a therapeutic nucleic acid payload to the transduced cells, allowing the sustained production of anti-inflammatory products.

Invention's implementation

What's the market's need? The idea of using cells for the delivery of disease-fighting drugs to specific sites in the body is not new, and many research groups and biotechnology companies are actually pursuing it. Despite considerable efforts to address the "drug delivery problem", delivering complex therapeutics, such as DNA, RNA, or ribonucleoprotein complexes, to specific cells of tissues in the body is very challenging.

How to address the identified need? I developed an invention that addresses the problems raised by viral, nanosystem-based, and cell-mediated drug delivery systems. The primary purpose of this invention is to deliver complex nucleic acid-encoded therapeutics to metastatic cancerous lesions. Of course, this delivery system has promising applications for the treatment of autoimmune and genetic diseases.

If successful, what will be the rewards? Drug development is a lengthy and risky process. To minimize risks and reduce the time from proof-of-concept study to reward, I envisage licensing this technology to industry partners, which are pharmaceutical and biotechnology companies. Each license will be exclusive and cover a defined target, which can be a tumor-associated antigen or a molecular vulnerability. Licensing fees are part of an upfront payment, which can reach $10-100 million. Prices are dictated by the number of indications (ex: lung, pancreatic, and ovarian cancers) that the treatment could cover, the results of preclinical efficacy and safety studies, and the treatment development stage.

According to J.P. Morgan & Co. since 2019, 90% of deals have happened in preclinical and earlier stages, and the median upfront cash payment and equity across 14 deals in 2022 is $115 million for cell therapy*.

*https://www.jpmorgan.com/content/dam/jpm/commercial-banking/insights/life-sciences/JPMorgan-Q1-2022-BioPharma-FINAL.pdf

Let's talk about this invention's feasibility. I conducted an exhaustive literature search and acquired advanced knowledge on DNA parts and plasmid design before deciding how to best implement this cell-mediated drug delivery system. I am "in possession" of all the parts required to construct this invention, making it 100% implementable. Details about my invention comprise: (a) the organization of the genetic circuit; (b) a compilation of DNA parts, including promoters, transgene's coding sequences, polyadenylation sequences, and recombinase binding sites; (c) a compilation of modules, which are an ordered assembly of DNA parts; and (d) a document that provides a detailed explanation of the general principle that governs my invention's working.

To obtain the delivery system, immune cells (T cells, NK cells, etc.) are transfected with a set of designed transposon plasmids, which will be stably integrated into the cells' genomes. Transposon vectors, co-transfected with transposase expressing vectors, are a well-established DNA transfer vehicle for integrating large constructs into a cell's genome [138,139]. Critical plasmid components must be tested for functionality—to verify that they work as expected—before they are incorporated into the designed plasmids.

The delivery system will be tested in vitro to assess its specificity and effectiveness. It should only produce and release a significant amount of viral vectors when co-cultured with cells that bear target antigens on their surfaces. The viral vectors contain payloads that encode a reporter protein, which will cause the transduced cells to fluoresce as a demonstration of successful payload delivery. If in vitro studies are satisfactory, the delivery system will be tested in animal models (ex: laboratory mice implanted with tumor xenografts).

We must obtain a patent for this invention to secure exclusivity. When preclinical data are obtained and patents are secured, the delivery system will be licensed to industry partners.

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Conflict of interest statement

The author declares no conflict of interest.

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